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(71) Applicants (for all designated States except US): NOVO INDUSTRI A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK). ZYMOGENETICS, INC. [US/US]; 4225 Roosevelt Way NE, Seattle, WA 98105 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only) : NICOLAISEN, Else, Marie [DK/DK]; Mariendalsvej 23A, lejl. 502, DK-2000 Frederiksberg (DK). BJØRN, Søren, Erik [DK/DK]; Marie Grubbes Allé 47, DK-2800 Lyngby (DK). WIBERG, Finn, Christoph [DK/DK]; Bybækterrasserne 151 D, DK-3520 Farum (DK). WOODBURY, Richard [US/US]; 15464 10th Avenue NE, Seattle, WA 98155 (US).

(74) Common Representative: NOVO INDUSTRI A/S; Novo Allé, DK-2880 Bagsværd (DK).

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(54) Title: MODIFIED FACTOR VII/VIIa

(57) Abstract

Modified factor VII/VIIa stabilized against proteolytic cleavage at certain positions in the molecule is provided. The stabilization is obtained by replacement of one or more proteolytically sensible peptide bonds in native human factor VII/VIIa with a proteolytically more stable peptide bond. Preferably certain Arg and/or Lys residues are replaced with other amino acids.

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MODIFIED FACTOR VII/VIIa

TECHNICAL FIELD

The present invention is related to modified factor VII/VIIa's, DNA sequences coding for such modified factors and a process for their production.

BACKGROUND ART

Factor VIIa is a serine protease that participates in blood coagulation by activating factor X and/or factor IX. Factor VIIa is produced from its precursor, factor VII, which is synthesized in the liver and secreted into the blood where it circulates as a single-chain glycoprotein (Mw = 50,000). Factor VII can in vitro be converted into the two-chain form factor VIIa by factor Xa, factor XIIa, factor IXa or thrombin. In the presence of tissue factor and calcium ions, factor VIIa, in vivo is believed to convert factor X to factor Xa by limited proteolysis. The latter enzyme in turn converts prothrombin to thrombin in the presence of factor Va, calcium ions, and phospholipid. Factor VIIa will also convert factor IX to factor IXa in the presence of tissue factor and calcium.

Factor VII can be purified from plasma and activated into factor VIIa by the methods described by Broze and Majerus, J. Biol.Chem. 255 (4): 1242 - 1247, 1980 and Hedner and Kisiel, J.Clin.Invest. 71: 1836 - 1841, 1983.

Factor VIIa may also be produced by recombinant DNA-technology by culturing in an appropriate medium mammalian cells transfected with a DNA-sequence encoding factor VII, isolating the protein produced and activating said protein to factor VIIa (vide European patent application No. 86302855.1).

The cDNA coding for human factor VII has been characterized (Hagen et al., Proc.Natl.Acad.Sci. USA, 83: 2412 - 2416, 1986). The amino acid sequence deduced from the cDNAs indicates that factor VII is synthesized with a prepro-leader sequence of 60 or 38 amino acids. The mature factor VII that circulates in plasma is composed of 406 amino acid residues. The amino acid sequence analysis of the activated protein and the amino acid sequence deduced from the cDNAs indicate that factor VII is converted to factor VIIa by the cleavage of a single peptide bond between arginine (152) and isoleucine (153). This results in the formation of a two-chained molecule consisting of a light chain (152 amino acid residues) and a heavy chain (254 amino acid residues) that are held together by one disulphide bond. The light chain contains a γ-carboxyglutamic acid (Gla) domain and two potential epidermal growth factor domains, while the heavy chain contains the serine protease portion of the molecule.

Factor VIIa may be used in treating patients who have developed inhibitors to factor VIII (Hedner, U. and Kisiel, W, J.Clin.Invest., 71: 1836 - 1841, 1983) and for the treatment of patients suffering from bleeding disorders such as platelet disorders including thrombocytopenia, von Willebrand's disease and others typically present in association with severe tissue damages (European patent application No. 86309197.1).

According to observations of the inventors hereof factor VIIa has been found to be a protein susceptible to proteolytic cleavage giving rise to a number of degradation products without clotting activity. The proteolytic cleavage may occur at different steps of the recovery procedure and also during storage. Degradation products have been observed both for factor VIIa derived from plasma as well as for factor VIIa produced by recombinant DNA-technology. The degradation may occur before factor VII has been activated into factor VIIa, i.e. during production and

isolation of factor VII, during the activating step itself or during isolation, purification and/or storage of the activated product.

As the degradation products are inactive molecules their occurrence in the factor VIIa preparation will lead to a lower specific activity of the final preparation. Furthermore, the amount and nature of the degradation products may vary from one production batch to another giving rise to preparations with a variable content of biologically active factor VIIa.

Factor VIIa preparations containing inactive degradation products will as mentioned have a less specific activity as compared to preparations in which all or a major part of the protein material is active. Accordingly, higher and more frequent doses are necessary to obtain and sustain a therapeutic or prophylactic effect as compared to a preparation with higher specific activity.

Variable amounts of inactive degradation products and as a consequence variable content of biologically active factor VIIa will furthermore make calculation of appropriate doses troublesome and difficult, if not in some circumstances impossible.

Finally, a content of non-physiological degradation products in the final preparation may trigger the immune system of the patient. Readministration may then result in allergic reactions, which in severe cases may have a lethal course. Patients may also develop high titers of antibodies against factor VIIa rendering subsequent treatment difficult or ineffective. Accordingly, a factor VIIa preparation with less tendency to proteolytic degradation in vitro will be more satisfactory and potentially more useful in factor VIIa therapy.

Factor VIIa is probably, like other circulating proteins, removed from the bloodstream by means of enzymatic degradation. In the initial step of this regulatory process the biologically active enzyme is cleaved at one or a few sensitive peptide bonds to produce

an inactive degraded molecule. It is very likely that the peptide bonds which are the most sensitive to enzymatic hydrolysis in vivo are identical to the labile peptide bonds which are most frequently observed to be hydrolyzed during production, purification and/or storage of factor VIIa (George J. Broze, Jr., Scot Hichman and Joseph P. Miletich, J.Clin.Invest. 76 (1985) 937-946).

Factor VII contains 17 lysine (positions 18, 32, 38, 62, 85, 109, 137, 143, 148, 157, 161, 197, 199, 316, 337, 341, 389) and 24 arginine (positions 9, 15, 28, 36, 79, 110, 113, 144, 152, 202, 223, 224, 247, 266, 271, 277, 290, 304, 315, 353, 379, 392, 396, 402) residues that in principle all are susceptible to proteolytic degradation, but usually a number of these residues are not "active" as cleaving sites.

Although the exact halflife of circulating factor VIIa is unknown, preliminary results suggest that factor VIIa procoagulant activity is rapidly cleared from the bloodstream upon intravenous administration (Ulla Hedner and Walter Kisiel, J.Clin.Invest. 71 (1983) 1836-1841).

The treatment and the lives of the patients will be negatively influenced by the observed short in vivo half life of native factor VIIa. Relatively high doses and frequent administration will be necessary to reach and sustain the desired therapeutic or prophylactic effect. As a consequence adequate dose regulation will be difficult to obtain and the need for frequent intravenous administrations will impose restrictions on the patients' way of living.

Consequently, there exists a need in the art for factor VIIa preparations which are stable during production, purification and storage even at high concentrations, and which furthermore have a longer half life and slower clearance from the blood than the native or recombinant factor VIIa. The present invention fulfills this need by providing certain modified factor VII/VIIa.

DESCRIPTION OF THE INVENTION

In its broadest aspect the present invention provides a modified factor VII/VIIa being stabilized against proteolytic cleavage at certain positions in the molecule. More specifically the present invention provides modified factor VII/VIIa in which one or more proteolytically sensible peptide bond(s) in native factor VII/VIIa has/have been replaced by a proteolytically more stable peptide bond.

According to the present invention this is achieved by modifications at certain positions in the native human factor VII/VIIa molecule. Such modifications may include removal of certain amino acid residues or replacement of one or more amino acid residues with a different amino acid residue. For instance a trypsin like proteolytic cleavage may be hindered by stabilizing the peptide bond on the C-terminal end of certain Arg and/or Lys residues and/or by replacement of certain Arg and/or Lys residues with other amino acid residues and/or by removal of certain Arg and/or Lys residues.

Examples of trypsin-like cleavage sites within the human factor VII molecule at which cleavages have been observed include

(i) lysine(38)-leucine(39),
(ii) lysine(32)-aspartate(33),
(iii) lysine(143)-arginine(144),
(iv) arginine(290)-glycine(291),
(v) arginine(315)-lysine(316),
(vi) lysine(316)-valine(317),
(vii) lysine(341)-glycine(342),
(viii) arginine(392)-serine(393),
(ix) arginine(396)-proline(397) and
(x) arginine(402)-alanine(403).

Minor chymotrypsin-like cleavages have also been observed after

(xi) isoleucine(42) and

(xii) tyrosine(44).

Of these the cleavage sites (i), (ii), (iv) and (v) have been found to be the ones most susceptible to proteolytic degradation, while the remaining are of less quantitative importance.

When considering the stabilization of factor VII/VIIa it is an important aspect that the resulting modified factor VII should retain its activity. This is according to the invention obtained by comparing the sequence of native factor VII/VIIa in the area to be modified with corresponding sequences in related proteins such as factor IX, factor X, factor II and protein C. Homologue sequences around the major cleavage sites are shown below:

```
                      32    38
                       |     |
Factor II      EEAFEALESSTATDVFWAKY
Factor VII     EEAREIFKDAERTKLFWISY
Factor X       EEAREVFEDSDKTNEFWNKY
Factor IX      EEAREVFENTERTTEFWKQY
Protein C      EEAKEIFQNVDDTLAFWSKH

                                290
                                 |
Factor II      RVTGWGNLKETWTANVGKGQPSV-L
Factor VII     LVSGWGQL-------LDRGATALEL
Factor X       IVSGFGRT-------HEKGRQSTRL
Factor IX      YVSGWGRV-------FHKGRSALVL
Protein C      LVTGWGYH-------SSREKEAKRN

                   315                           341
                    |                             |
Factor II      C-KDSTRI----RITDNMFCAGYKPDEGKRGDACEGDSGGPF
Factor VII     CLQQSRKVGDSPNITEYMFCAGYS--DGSK-DSCKGDSGGPH
Factor X       C-----KLSSSFIITQNMFCAGYD--TKQE-DACQGDSGGPH
Factor IX      CLR-STKFT----IYNNMFCAGFH--EGGR-DSCQGDSGGPH
Protein C      CSEVMSNH-----VSENMLCAGIL--DGRQ-DACEGDSGGPM
```

Consequently, it is an object of the present invention to provide for modified factor VII/VIIa wherein one, more or all of the lysine, arginine, isoleucine and tyrosine residues:

(i) lysine(38)
(ii) lysine(32)
(iii) lysine(143)
(iv) arginine(290)
(v) arginine(315)
(vi) lysine(316)
(vii) lysine(341)
(viii) arginine(392)
(ix) arginine(396)
~~(x) arginine(402)~~
(xi) isoleucine(42) and
(xii) tyrosine(44)

have been stabilized by substitution or deletion.

In a preferred embodiment of the invention one, more or all of the amino acid residues in positions (32), (38), (290) and (315) have been stabilized by substitution or deletion.

According to the present invention Lys in position 32 (ii) and/or 38 (i) may be replaced by another amino acid residue. Lys(38) may preferably be replaced by Thr, Asp, Leu, Gly, Ala, Ser, Asn or His and Lys(32) may preferably be replaced by Gln, Glu, His, Gly, Thr, Ala, or Ser.

Also Arg in position 290 (iv) may be replaced by another amino acid residue, for instance Gly, Ala, Ser, Thr or Lys, preferably Ser, Ala or Gly.

Arg(315) (v) may preferably be substituted by Gly, Thr, Ala, Ser or Gln.

Furthermore Lys(341) (vii) may be substituted by Glu, Gln, Gly, Thr, Ala or Ser, preferably Glu or Gln.

Besides substitution of the above mentioned Arg respective Lys residues with another amino acid residue removal of the Arg or Lys amino acid residues may also be considered in order to avoid proteolytic cleavage. Furthermore, one or more of the amino acid residues on either the N- or C-terminal side of such Arg or Lys residues may be substituted by another amino acid residue

exerting a stabilizing effect on the proteolytically sensible peptide bond. An example of such modifications is substitution of the amino acid residue linked to the C-terminal end of a Lys or Arg residue with Pro.

To avoid proteolytic cleavage at position 42 (xi) and 44 (xii), Ile(42) and/or Tyr(44) may be substituted by Asn, Ser, Ala or Gln.

The present invention is contemplated to cover any combination of the above mentioned substitutions and deletions.

Other aspects of the invention will become evident upon reference to the following detailed description and attached drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the amino acid sequence given by one letter abbreviations and the tentative structure for factor VII,

Figure 2 illustrates the construction of plasmid pFW10-3/6,

Figure 3 illustrates the construction of plasmid pFW60-3/6, and

Figure 4 illustrates the construction of plasmid pFWx-3/6.

DEFINITIONS

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

Complementary DNA or cDNA: A DNA molecule or sequence which has been enzymatically synthesized from the sequences present in a mRNA template or a clone of such molecule.

DNA Construct: A DNA molecule, or a clone of such a molecule, either single- or double-stranded, which may be isolated in partial form from a naturally occurring gene or which has been modified to contain segments of DNA which are combined and juxtaposed in a manner which would not otherwise exist in nature.

Plasmid or Vector: A DNA construct containing genetic information which may provide for its replication when inserted into a host cell. A plasmid generally contains at least one gene sequence to be expressed in the host cell, as well as sequences encoding functions which facilitate such gene expression, including promoters and transcription initiation sites. It may be a linear or closed circular molecule. As used herein, the term expression vector shall mean a plasmid or vector containing a transcription promoter and terminator operably linked to a DNA sequence encoding a protein or polypeptide of interest. Expression vectors may further contain other elements, including selectable markers, enhancers, polyadenylation signals, etc., which will be determined in part by the particular host cell chosen.

Biological Activity: A function or set of functions performed by a molecule in a biological context (i.e. in an organism or an in vitro facsimile). Biological activities of proteins may be divided into catalytic and effector activities. Catalytic activities of clotting factors generally involve the activation of other factors through the specifid cleavage of precursors. Effector activities include specific binding of the biologically active molecule to calcium or other small molecules, to

macromolecules such as proteins or to cells. Effector activity frequently augments, or is essential to, catalytic activity under physiological conditions. Catalytic and effector activities may, in some cases, reside within the same domain of a protein.

For factor VIIa biological activity is characterized by the mediation of blood coagulation through the extrinsic pathway. Factor VIIa activates factor X to factor Xa, which in turn converts prothrombin to thrombin thereby initiating the formation of a fibrin clot.

The modified factor VIIa according to the present invention has a biological activity that is substantially the same as that of native factor VIIa.

"Factor VII/VIIa" as used in this application means a product consisting of either the unactivated form (factor VII) or the activated form (factor VIIa) or mixtures thereof. "Modified factor VII/VIIa" shall mean a biologically active molecule derived from factor VII/VIIa by the substitution or deletion of one or more amino acid residues.

As the modifications according to the present invention is made on gene expression level modifications introduced in the factor VII molecule will also be found in the activated product (factor VIIa).

"Factor VII/VIIa" within the above definition includes proteins that have the amino acid sequence of native human factor VII/VIIa. It also includes proteins with a slightly modified amino acid sequence for instance a modified N-terminal end including N-terminal amino acid deletions or additions so long as those proteins substantially retain the activity of factor VIIa.

"Factor VII" within the above definition also includes natural allelic variations that may exist and occur from one individual to another. Also degree and location of glycosylation or other post-translation modifications may vary depending on the chosen host cells and the nature of the host cellular environment.

The number system of the amino acid sequence of factor VII/VIIa used herein appears from Figure 1 in which the N-terminal alanine is numbered 1 and the C-terminal proline is numbered 406.

The three letter and one letter abbreviations used for the amino acids are those as normally used in the art, i.e.:

Amino acid	Three letter abbreviation	One letter abbreviation
Alanine	Ala	A
Cysteine	Cys	C
Asparatate	Asp	D
Glutamate	Glu	E
Phenylalanine	Phe	F
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Lysine	Lys	K
Leucine	Leu	L
Methionine	Met	M
Asparagine	Asn	N
Proline	Pro	P
Glutamine	Gln	Q
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V
Tryptophan	Trp	W
Tyrosine	Tyr	Y
γ-carboxyglutamic acid	Gla	γ

Best Mode for Carrying out the Invention

The amino acid changes are preferably introduced by oligonucleotide-directed site specific mutagenesis in factor VII cDNA. After screening E. coli cells the mutated factor VII gene is isolated and recloned into a suitable expression vector. The expression vector is then transfected into an appropriate host cell which when cultured in a suitable culture medium expresses and secretes the modified factor VII which after recovery from the culture medium is converted into the corresponding modified factor VIIa by known means.

Various host cells may be used including mammalian cells, yeast and other fungi, and bacteria. However, mammalian cells are preferred. A particularly preferred mammalian cell line is the BHK cell line $tk^{-}ts13$ (Waechter and Basserga, Proc.Natl.Acad.Sci. USA 79: 1106-1110, 1982). Methods for expressing cloned genes in each of these hosts are known in the art, vide for instance EP published patent application No. 200,421 (expression of factor VII and IX in mammalian cells), EP published patent application No. 191,606 (expression of protein C in bacterial cells and EP published patent application No. 167,420 (expression of factor IX in yeast).

For expression of modified factor VII according to the invention in cultured mammalian cells, expression vectors containing cloned modified factor VII sequences are introduced into the cells by appropriate transfection techniques, such as calcium phosphate-mediated transfection (Graham and Van der Eb, Virology 52: 456-467, 1973; as modified by Wigler et al., Proc.Natl.Acad.Sci. USA 77: 3567-3570, 1980). Electroporation transfection technique may also be used (Neuman et al., EMBO.J. 1: 841-845, 1982). A DNA-calcium phosphate precipitate is formed, and this precipitate is applied to the cells. A portion of the cells take up the DNA and maintain it inside the cell for several

days. A small fraction of the cells integrate the DNA into the genome of the host cell. These integrants are identified by cotransfection with a gene that confers a selectable phenotype (a selectable marker). A preferred selectable marker is the mouse dihydrofolate reductase (DHFR) gene, which imparts cellular resistance to the drug methotrexate (MTX). After the host cells have taken up the DNA, drug selection is applied to select for a population of cells that are expressing the selectable marker in a stable fashion.

Modified factor VII produced by the transfected cells may be removed from the cell culture media by adsorption to barium citrate. Spent medium is mixed with sodium citrate and barium chloride and the precipitate collected. The precipitated material may then be assayed for the presence of the appropriate clotting factor. Further purification may be achieved through immunoadsorption. It is preferred that the immunoadsorption column comprise a high-specificity monoclonal antibody. Alternatively, purification of the barium citrate precipitated material may be accomplished by more conventional biochemical methods or by high-performance liquid chromatography (HPLC).

Conversion of single-chain modified factor VII to active two-chain modified factor VIIa may be achieved using factor XIIa as described by Hedner and Kisiel (J.Clin.Invest. 71: 1836-1841, 1983), or with other proteases having trypsin-like specificity (Kisiel and Fujikawa, Behring Inst. Mitt. 73: 29-42, 1983). Alternatively modified factor VII may be activated by passing it through an ion-exchange chromatography column, such as mono Q® (Pharmacia Fire Chemicals) or the like (Bjoern et al., Research Disclosures, 269, September 1986, pp. 564 - 565).

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

MATERIALS AND METHODS

Restriction enzymes were obtained from Bethesda Research Laboratories (BRL), New England Biolabs, and Stratagene and were used as indicated by the producer, unless otherwise stated, Oligonucleotides were synthesized on an automatic DNA synthesizer using phosphoramidite chemistry on a controlled pore glass support (S.L. Beaucage and M.H. Caruthers (1981) Tetrahydron Letters 22, 1859 - 1869). *E. coli* cells were transformed as described by Maniatis et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory, 1982).

A representative modified factor VII was prepared by changing amino acid No. 32 from Lys → Gln and amino acid No. 38 from Lys → Thr by oligonucleotide directed mutagenesis.

The oligonucleotides with these changes are of the sequences:

```
I)                               BglII      Lys(32)

cDNA:                      ... ... GAG ... ... AAG ... ... ...
                                    ↓           ↓
oligonucleotide: 5' GCC CGG GAA ATC TTC CAG GAC GCG GAG 3'
                                    ↑           ↑
     nucleotide position           228         235
                                   Glu         Gln
```

which is a 27-mer with changes at nucleotide position 228 which destroy a BglII site without changing the amino acid and at position 235 changing amino acid Lys(32) to Gln.

```
II)                              Lys(38)

cDNA                   ... ... AAG ... ... ... ...
                                ↓
oligonucleotide: 5' AGG ACG ACG CTG TTC TGG ATT 3'
                                ↑
     nucleotide position        254
                                Thr
```

which is a 21-mer with changes at nucleotide position 254 changing amino acid Lys(38) to Thr.

A further representative modified factor VII was prepared by changing amino acid No. 290 from Arg -- Ser and amino acid No. 315 from Arg -- Ser by oligonucleotide directed mutagenesis..

The oligonucleotides with these changes are of the sequences:

```
(III)                                Arg

cDNA:                  ...  ... ... CGT ... ... ... ... ..
                                     ↓
oligonucleotide: 5' GCTG CTG GAC AGT GGC GCC ACG GCC CT
                                     ↑
     nucleotide position             1009

                                     Ser
```

which is a 27-mer with changes at nucleotide position 1009 changing the amino acid Arg (290) to Ser.

```
(IV)                              Arg

cDNA                 .... ... ... CGG ... ... ... ... .
                                  ↓ ↓
oligonucleotide: 5' GCAG CAG TCA AGT AAG GTG GGA GAC T
                                  ↑ ↑
     nucleotide position:       1084 1086

                                  Ser
```

which is a 26-mer with changes at nucleotide position 1084 and 1086 changing amino acid Arg(315) to Ser.

<u>Example 1</u>

Production of a modified factor VIIa in which Lys(32) has been replaced with Gln (factor VIIa(Gln(32)).

Recloning Factor VII cDNA

Factor VII cDNA with a 38 amino acid long leader (Berkner, K.L. et al., Cold Spring Harbor Symposium on Quantitative Biology, Vol. LI, 531-541, 1986) was cloned in the EcoRI site of pGEM3 vector (Promega Biotec) and propagated in <u>E. coli</u> MC 1061 (dam^+) or MC 1000 (dam^-) bacteria strain.

Briefly, plasmid FVII(565 + 2463)/pDK was cut with EcoRI and the factor VII cDNA was ligated to EcoRI cut pGEM3. The construction of plasmid FVII(565 + 2463)/pDX is described in EP patent application No. 86302855.1. The plasmid has also been deposited at American Type Culture Collection (ATTC No. 40205).

Small and large scale DNA preparations were prepared as described in for example Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbour 1982. One of these plasmid preparations is termed pFW 10-3/6. The construction of pFW 10-3/6 is illustrated in fig. 2.

Addition of 5' phosphate groups to oligonucleotides

Addition of either labelled or unlabelled phosphate groups to oligonucleotides was carried out as described (Maniatis et al. as above).

Oligonucleotide directed site specific mutagenesis using double-stranded plasmid DNA

The site directed mutagenesis reaction was carried out by modifying the method by Morinaga et al. 1984 (BIO/TECHNOLOGY vol. 2 p. 636). Plasmid pFW10-3/6 containing FVII cDNA was digested with BglI, a unique site in the plasmid. This cleavage generated fragment a) shown in fig. 3 and fig. 4 destroying the ampicillin resistance. Fragment a) was purified by electroelution from agarose gel and treated with calf intestinal alkaline phosphatase (CIAP) as described in Maniatis et al. as above.

Another sample of pFW 10-3/6 was digested with BssHII and SacII generating fragment b) in fig. 3 with a window of 575 bp in the FVII cDNA. Fragment b) was purified by electroelution from agarose gel after electrophoresis.

Fragments a) and b) were further purified by several phenol extractions, phenol/chloroform (1:1, v/v) and chloroform/isoamyl alcohol (24:1, v/v) extractions, precipitated with 0.3 M Na-acetate and 70% (v/v) ethanol and dissolved in TE (10 mM Tris, 1 mM EDTA pH 7.6).

Then 0.1 - 0.15 pmol of both fragment a) and b) were mixed with 25 pmol of the phosphorylated synthetic oligonucleotide I) in an Eppendorf tube.

Then 10 µl of 5Xpolymerase-ligase buffer (0.5 M NaCl, 33 mM Tris HCl pH 7.5, 40 mM $MgCl_2$, 5 mM 2-ME) were added.

From the final mixture 15 µl samples were removed and stored on ice until later use as marker in gel electrophoresis. The remaining mixture was incubated in a boiling water bath for 4 min. to denature the DNA fragments. After incubation the mixture was gradually cooled. Upon reannealing heteroduplexes were formed and using agarose gel electrophoresis the formation of a new circular DNA with the correct mutation was demonstrated by comparison with the non-heated sample from above.

Then 10 µl of the four deoxyribonucleoside triphosphate (2.5 mM each), 3 µl of 20 mM ATP, 1 µl of Klenow fragment of DNA polymerase I (5 U/µl) and 1 µl of T4 DNA ligase (10 U/µl) were added to the mixture (20 µl) of heteroduplexes (final volume 40 µl). The final mixture was incubated at 12°C overnight.

Transformation of E. coli MC 1061 and MC 1000 with the incubation mixture resulted in ampicillin resistant transformants. Transformants carrying the mutant FVII gene were selected by colony hybridization (Maniatis et al.) with the 5'-^{32}P-labelled 27-mer and 21-mer synthetic oligonucleotides.

After retransformation plasmid DNA was purified from selected colonies, analysed, and sequenced (by the Maxam-Gilbert method and the dideoxy method) to verify the mutation caused by the synthetic oligonucleotide.

The construction of plasmid pFW 60-3/6 harbouring a mutated factor VII gene in which Lys(32) has been replaced with Gln is illustrated in fig. 3.

pFW 60-3/6 was digested with EcoRI and the EcoRI-EcoRI factor VII fragment was ligated into EcoRI cut pDx plasmid to obtain plasmid pFW 78-3/6 harbouring the factor VII(Gln32) gene in the same orientation as in

plasmid FVII(565 + 2463)/pDX. Plasmid pFW 78-3/6 was then transfected into BHKtk^{-}ts13 cells following the general procedure described above.

The modified factor VII produced by the cells is then precipitated with barium citrate; purified by immunoadsorption; and activated to modified factor VIIa by passing it through an ion-exchange chromatography column as described by Bjoern et al., supra.

Test for Activity

As activated native factor VIIa, the activated modified factor VIIa shortened the coagulation period in a one-stage clotting assay. The activated modified factor VIIa was incubated at a concentration of approximately 0,9 mg/ml in a 10 mM Tris-HCl buffer at pH 8.5 comprising 390 mM NaCl and 5 mM EDTA. The degradation was monitored by SDS-PAGE of reduced samples and when significant degradation had occurred an aliqot was withdrawn and applied to an HPLC column. The preparative chromatography served mainly to exclude Tris from the sample for amino acid sequencing as intact and degraded modified factor VIIa coeluted from the column. N-terminal amino acid sequencing revealed that no hydrolysis had occurred of the peptide bond between glutamine residue No. 32 and aspartic acid residue No. 33. In contrast profound degradation at lysine residue No. 32 was observed when activated native factor VIIa was subjected to the same treatment and analysis as performed in a parallel investigation.

Example 2

Production of a modified factor VIIa in which Lys(38) has been replaced with Thr (factor VIIa(Thr 38)).

By following the procedure of example 1 with the only exception that the synthetic oligonucleotide II) was used instead of I) an expression plasmid was obtained harbouring the mutated factor VII gene.

This plasmid is then transfected into BHKtk$^-$ts13 cells and factor VII(Thr38) is recovered from the cell supernatant and activated to factor VIIa(Thr38) as described.

Example 3

Production of a modified factor VIIa in which Lys(32) and Lys(38) have been replaced with Gln and Thr, respectively (factor VIIa(Gln32, Thr38)).

By following the procedure of example 1 with the only exception that 12.5 pmol of both oligonucleotide I) and II) are used in the site directed mutagenesis reaction, an expression plasmid harbouring the mutated factor VII gene was obtained.

This plasmid is then transfected into BHKtk$^-$ts13 cells and factor VII(Gln32, Thr38) is recovered from the cell supernatant and activated to factor VIIa(Gln32, Thr38) as described.

Example 4

Production of a modified factor VIIa in which Arg(290) has been replaced with Ser (factor VIIa(Ser(290)).

The construction of plasmid pFW A-3/6 harbouring a mutated factor VII gene in which Arg(290) has been replaced with Ser is illustrated in fig. 4.

Plasmid pFW 10-3/6 was used to produce the fragment a) of Example 1; and another sample of pFW 10-3/6 was digested with SacII and DraIII generating fragment b) in fig. 4 with a window of 1366 bp in the FVII cDNA.

Fragments b) and a) were subsequently treated as described in Example 1 except for the use of oligonucleotide III) instead of I).

pFW A-3/6 was digested with EcoRI and the EcoRI-EcoRI factor VII fragment was ligated into EcoRI cut pDx plasmid to obtain plasmid pFW X-3/6 harbouring the factor VII(Ser290) gene in the same orientation as in plasmid FVII(565 + 2463)/pDX. Plasmid pFW X-3/6 was then transfected into BHKtk$^-$ts13 cells following the general procedure described above.

The modified factor VII produced by the cells is then precipitated with barium citrate; purified by immunoadsorption; and activated to modified factor VIIa by passing it through an ion-exchange chromatography column as described by Bjoern et al., supra.

Example 5

Production of a modified factor VIIa in which Arg(315) has been replaced with Ser (factor VIIa(Ser315)).

By following the procedure of example 4 with the only exception that the synthetic oligonucleotide IV) was used instead of III) an expression plasmid was obtained harbouring the mutated factor VII gene.

This plasmid is then transfected into BHKtk$^-$ts13 cells and factor VII(Ser315) is recovered from the cell supernatant and activated to factor VIIa(Ser315) as described.

Example 6

Determination of Three Active Proteolytic Cleavage Sites

In order to identify some active cleavage sites a heavily degraded preparation of recombinant factor VII was submitted to N-terminal sequence analysis by automated Edman degradation using an Applied Biosystems model 470 A gas-phase sequencer. The results are shown in Table 1 below

Table 1

Cycle No.	PTH-a.a.	Yield (pmol)	PTH-a.a.	Yield (pmol)	PTH-a.a.	Yield (pmol)	PTH-a.a.	Yield (pmol)
1	Leu	593	Ile	756	Gly	706	Lys	425
2	Phe	497	Val*	1183	Ala	232	Val*	1183
3	Trp	341	Gly*	1128	Thr	38	Gly*	1128
4	Ile	458	Gly	780	Ala	166	Asp	413
5	Ser*	213	Lys	936	Leu	262	Ser*	213
6	Tyr	397	Val	885	Glu	154	Pro	386
7	Ser	94	(1/2Cys)	n.d.	Leu	240	Asn**	n.d.
8	Asp	312	Pro	690	Met	136	Ile	416
9	Gly	400	Lys	815	Val	289	Thr	104
10	Asp	278	Gly	647	Leu	225	Glu	354
11	Gln	274	Glu	670	Asn	122	Tyr	389
12	(1/2Cys)	n.d.	(1/2Cys)	n.d.	Val	267	Met	363
13	Ala	379	Pro*	604	Pro*	604	Phe	370
14	Ser	31	Trp	8	Arg	270	(1/2Cys)	n.d.
15	Ser	66	Gln	394			Ala	427

(*) The total amount of amino acid residues occuring from two sequences is given

(**) Glycosylated Asn

n.d. Not determined

In this sample four N-terminals were deduced Leu-39 (column 1), Gly-291 (column 3) and Lys-316 (column 4) corresponding to proteolytic cleavage and Ile-153 (column 2) corresponding to activation of FVII to FVIIa.

CLAIMS

1. Modified factor VII/VIIa, wherein one, more or all the lysine, arginine, isoleucine, and tyrosine residues

(i) lysine(38)
(ii) lysine(32)
(iii) lysine(143)
(iv) arginine(290)
(v) arginine(315)
(vi) lysine(316)
(vii) lysine(341)
(viii) arginine(392)
(ix) arginine(396)
(x) arginine(402)
(xi) isoleucine(42) and
(xii) tyrosine(44)

have been substituted and/or deleted.

2. Modified factor VII/VIIa according to claim 1, wherein one, more, or all the amino acid residues (i), (ii), (iv) or (v) have been substituted or deleted.

3. Modified factor VII/VIIa according to claim 1 or 2 wherein Lys(38) has been replaced with Thr, Asp, Leu, Gly, Ala, Ser, Asn or His.

4. Modified factor VII/VIIa according to claim 3, wherein Lys(38) has been replaced with Thr.

5. Modified factor VII/VIIa according to claim 1 or 2, wherein Lys(32) has been replaced with Gln, Glu, His, Gly, Thr, Ala or Ser.

6. Modified factor VII/VIIa according to claim 5 wherein Lys(32) has been replaced with Gln.

7. Modified factor VII/VIIa according to claim 1 or 2, wherein Arg(290) has been replaced by Gly, Ala, Ser, Thr or Lys.

8. Modified factor VII/VIIa according to claim 7, wherein Lys(38) has been replaced with Ser, Ala or Gly.

9. Modified factor VII/VIIa according to claim 1 or 2, wherein Arg(315) has been replaced by Gly, Thr, Ala, Ser or Gln.

10. Modified factor VII/VIIa according to claim 1 or 2, wherein wherein Lys(341) has been replaced by Glu, Gln, Gly, Thr, Ala or Ser, preferably Glu or Gln.

11. Modified factor VII/VIIa according to claim 1 or 2, wherein Asn, Ser, Ala, or Gln has been substituted for Ile(42).

12. Modified factor VII/VIIa according to claim 1 or 2, wherein Asn, Ser, Ala, or Gln has been substituted for Tyr(44).

13. Modified factor VII/VIIa according to claim 1 or 2, comprising any combination of the substitutions of any of the claims 3 to 12.

14. Modified factor VII/VIIa wherein Lys(38) has been replaced with Thr and Lys(32) has been replaced with Gln.

15. DNA-sequences encoding a modified factor VII according to any of claims 1-14.

16. Expression vectors containing a DNA-sequence according to claim 15.

17. Cells transformed to produce a modified factor VII according to any of claims 1-14.

18. A method for the production of modified factor VII/VIIa, wherein a cell transformed with an expression vector containing a DNA-sequence which codes for the modified factor VII/VIIa is cultured in an appropriate medium, the modified factor VII encoded by said DNA-sequence is isolated, and optionally is activated to generate a modified factor VIIa.

1/4

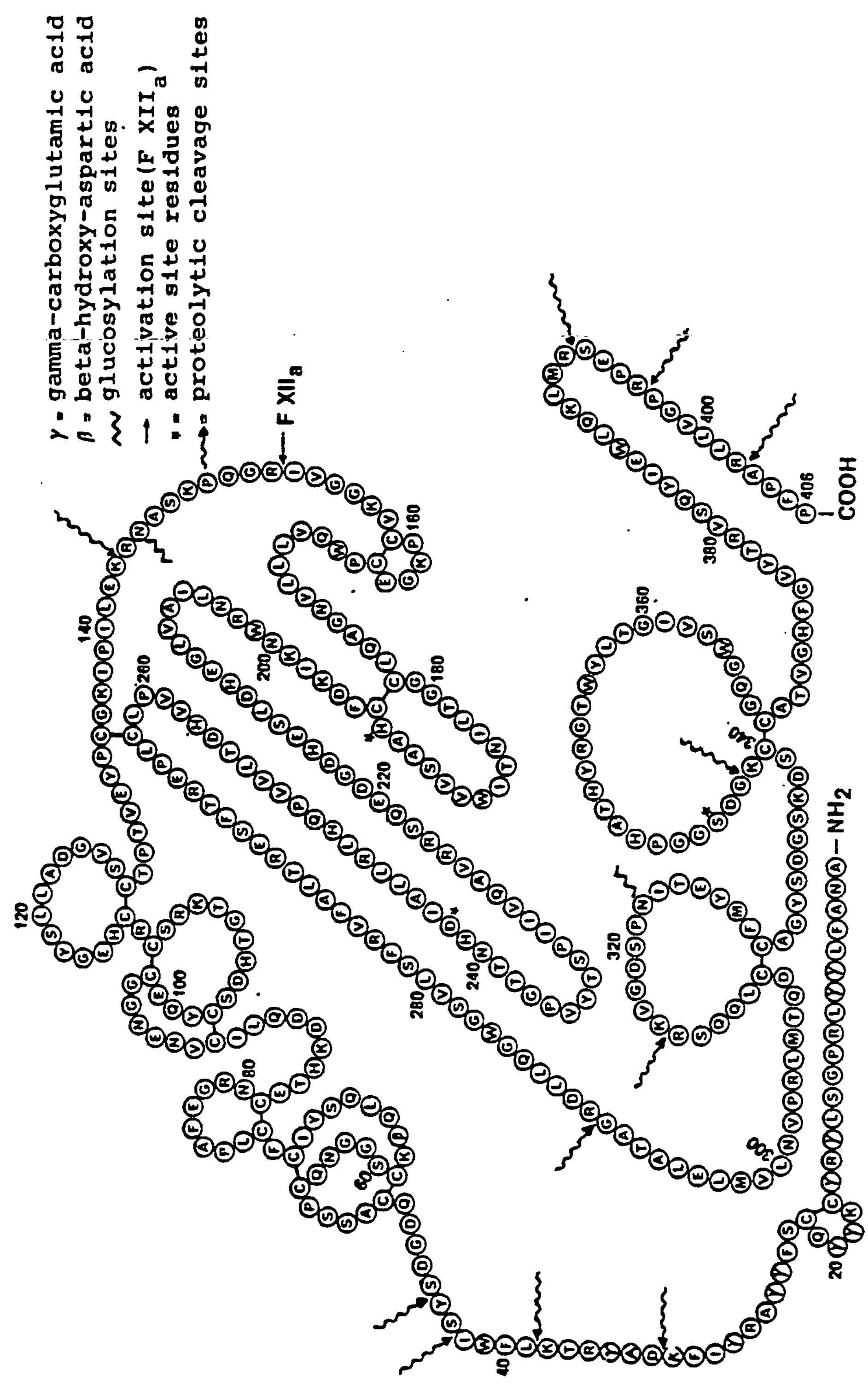
γ = gamma-carboxyglutamic acid
β = beta-hydroxy-aspartic acid
glucosylation sites
activation site(F XII_a)
* = active site residues
proteolytic cleavage sites
F XII_a
COOH
NH_2

FIG. 1

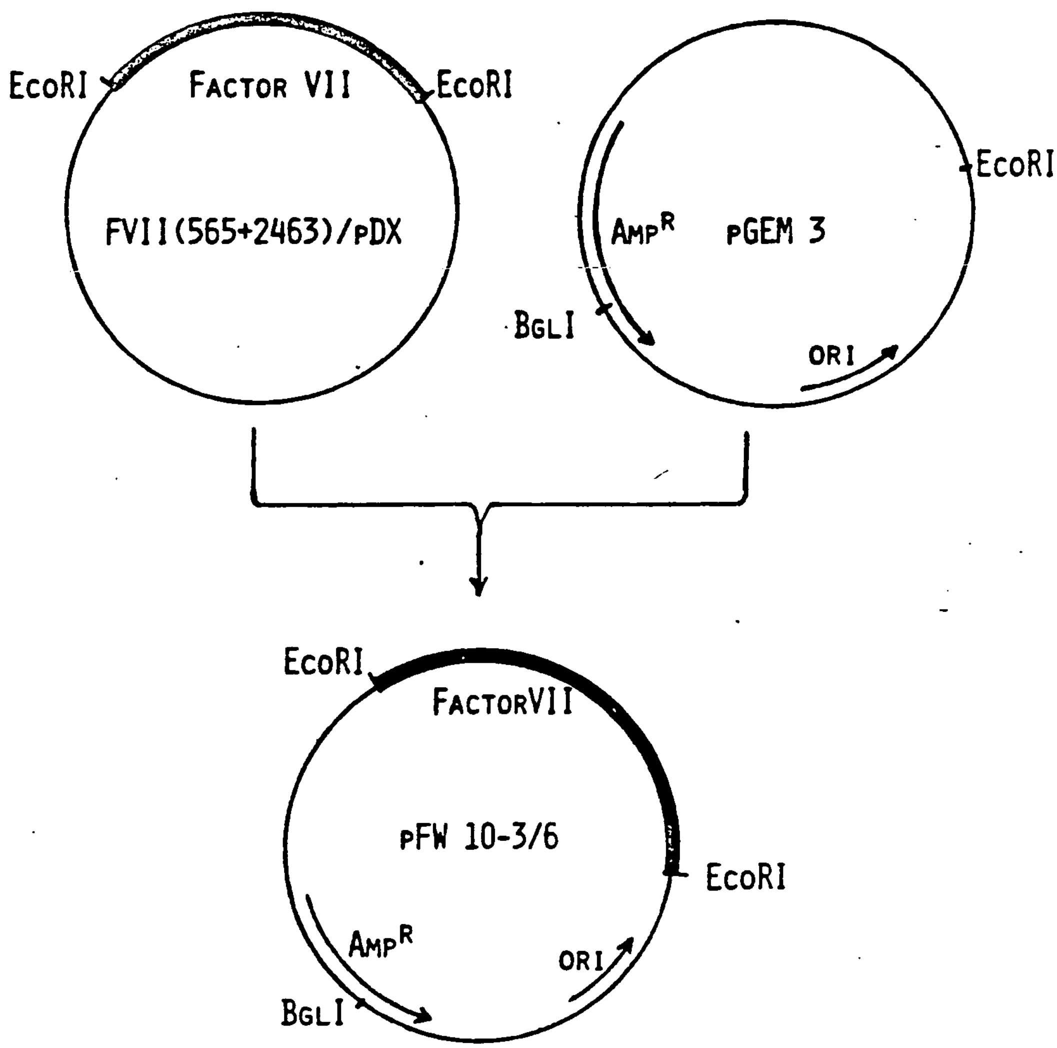
EcoRI
FACTOR VII
EcoRI
FVII(565+2463)/pDX
EcoRI
AMP^R
pGEM 3
BGLI
ORI
EcoRI
FACTORVII
pFW 10-3/6
EcoRI
AMP^R
ORI
BGLI

FIG. 2

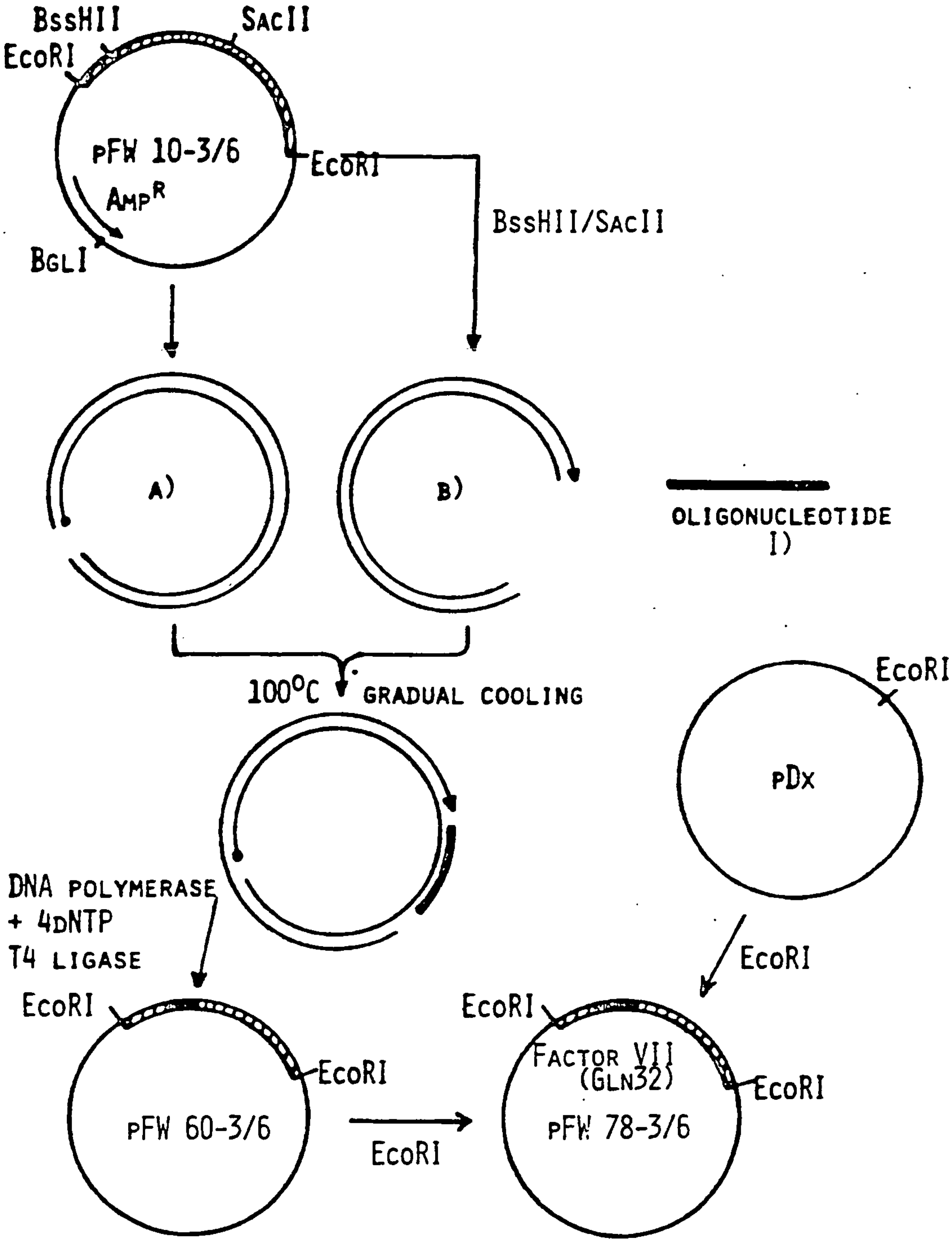
BssHII
SacII
EcoRI
pFW 10-3/6
AmpR
EcoRI
BglI
BssHII/SacII
A)
B)
OLIGONUCLEOTIDE
I)
100°C
GRADUAL COOLING
EcoRI
pDx
DNA polymerase
+ 4dNTP
T4 ligase
EcoRI
EcoRI
EcoRI
pFW 60-3/6
EcoRI
EcoRI
Factor VII
(Gln32)
EcoRI
pFW 78-3/6

Fig. 3

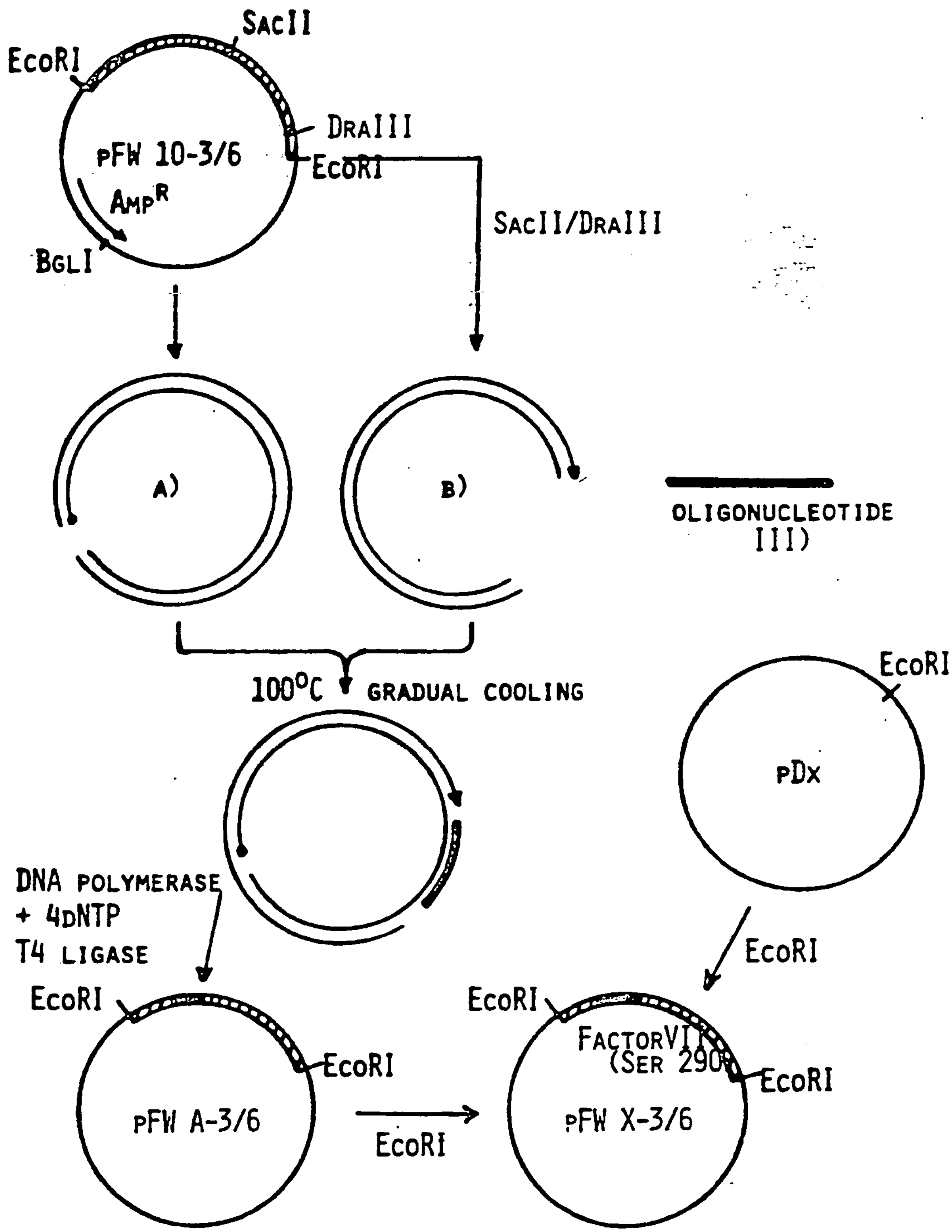
SacII
EcoRI
DraIII
pFW 10-3/6
EcoRI
Amp^R
BglI
SacII/DraIII
A)
B)
OLIGONUCLEOTIDE
III)
100°C
GRADUAL COOLING
EcoRI
pDx
DNA POLYMERASE
+ 4dNTP
T4 LIGASE
EcoRI
EcoRI
EcoRI
FactorVII
(Ser 290)
EcoRI
EcoRI
pFW A-3/6
EcoRI
pFW X-3/6

Fig. 4

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK88/00103

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC 4

C 12 N 9/50, C 12 N 15/00, C 12 P 21/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC 4	C 12 N 9/48, /50, /64, C 12 N 15/00
US Cl	424:94, 435:219, 226

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

SE, NO, DK, FI classes as above.
Databasesearch: WPIL, WPI, CA

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	EP, A, 0 200 421 (ZYMOGENETICS, INC.) 10 December 1986 & JP, 62000283	1-18
Y	Chemical Abstracts Vol. 106 (1987), abstract 106:46313q, Biochemistry (Tate K.), 1987, 26(2), 338-43 (Eng).	1-18
Y	EP, A, 0 201 153 (BEECHAM GROUP PLC) 12 November 1986 & JP, 61233630	1-18
Y	Chemical Abstracts Vol. 103 (1985), abstract 103:83655m, Proc. Natl. Acad. Sci. U.S.A. 1985, (Horwich A.L.), 82(15), 4930-3 (Eng).	1-18
Y	WO, A, 86/01538 (BIOGEN N.V.) 13 March 1986 & EP, 0191843	1-18
Y	Chemical Abstracts Vol. 105 (1986), abstract 105:2280b, FEBS Lett. 1986, (Schwartz T.), 200(1), 1-10 (Eng).	1-18

.../...

* Special categories of cited documents: [10]
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
1988-09-02	1988 -09- 19
International Searching Authority	**Signature of Authorized Officer**
Swedish Patent Office	Yvonne Siösteen Yvonne Siösteen

Form PCT/ISA/210 (second sheet) (January 1985)

International Application No. PCT/DK88/00103

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	J. Clin. Invest. Vol. 76, p. 937-946, published September 1985 (GEORGE J. BROZE, JR. et al) "Monoclonal Anti-human Factor VII Antibodies".	1-18
P Y	EP, A, 0 233 013 (BEECHAM GROUP PLC) 19 August 1987 & JP, 62236481	1-18

Form PCT ISA/210 (extra sheet) (January 1985)